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Original Article

High glucose-induced DNA-binding activities of nuclear factor of activated T cells 5 and carbohydrate response element binding protein to the myo-inositol oxygenase gene are inhibited by sorbinil in peripheral blood mononuclear cells from patients with type 1 diabetes mellitus and nephropathy

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ABSTRACT

Aims: To investigate whether high glucose induces myo-inositol oxygenase (MIOX) expression in peripheral blood mononuclear cells through transcription factors, nuclear factor of activated T cells 5 (NFAT5) and carbohydrate response element binding protein (ChREBP), which may contribute to the pathogenesis of diabetic nephropathy.

Methods: 34 patients with type 1 diabetes mellitus (20 with nephropathy, 14 without complications) and 9 healthy controls were recruited in this study. Peripheral blood mononuclear cells were exposed to normal, high glucose conditions with/without an aldose reductase inhibitor (ARI), using electrophoretic mobility shift assays the DNA-binding activities of NFAT5 and ChREBP to corresponding sites in the promoter region of MIOX gene were analysed. The protein levels and the enzyme activity of MIOX were measured.

Results: DNA-binding activities of NFAT5 and ChREBP were increased under high glucose conditions and decreased in the presence of the ARI in all groups. In the presence of ARI, the DNA-binding activities of NFAT5 and ChREBP were significantly decreased by 41% (NFAT5: 0.91 ± 0.06 vs. 1.54 ± 0.12; $p = 0.01$) and 49% (ChREBP: 0.92 ± 0.08 vs. 1.81 ± 0.22; $p = 0.001$) compared with high glucose in patients with nephropathy. ARI treatment decreased the protein levels of MIOX under high glucose conditions in patients with nephropathy (0.81 + 0.19 vs. 1.3 + 0.04; $p = 0.049$).

Summary/conclusions: There was a trend for increased binding activities of NFAT5 and ChREBP accompanied with increased protein levels under high glucose, particularly in patients with nephropathy. ARI treatment prevented these increases and this effect was more obvious in the patients with nephropathy compared to the uncomplicated subjects.

1. Introduction

It has been widely accepted that long-term exposure to high blood glucose plays an important role in the development of diabetic nephropathy [1]. High glucose, together with increased flux through the polyol pathway causes myo-inositol (MI) depletion which may have a role to play in the development of diabetic nephropathy. However, the mechanism of MI depletion is still unclear. It has been proposed that myo-inositol oxygenase (MIOX) plays a key role in causing the depletion of MI.

There are two enzymes in the polyol pathway, aldose reductase (AKR1B1) and sorbitol dehydrogenase. Together, they convert glucose to fructose via sorbitol. Under physiological conditions, most of the cellular glucose is phosphorylated into glucose 6-phosphate, and only a minor portion is metabolised through the polyol pathway. However, high glucose conditions may saturate the enzymes involved in the phosphorylation of glucose, and as a result, one-third of intracellular glucose enters the polyol pathway, which causes an accumulation of sorbitol within cells. Accumulation of sorbitol is accompanied by a depletion of MI, and this alters Na^+/K^+ ATPase activity and impairs phosphatidylinositol syntheses, which is a common precursor of important secondary signalling molecules. Several studies have shown that MI depletion is associated with diabetic nephropathy, retinopathy, neuropathy and diabetic cataracts [2–7]. MIOX is the first and rate-limiting enzyme in the MI metabolism pathway, which is the glucuronate–xylulose pathway and is the only pathway for MI catabolism. It has been shown that an increase in MIOX enzyme activity is in proportion to serum glucose concentrations [8]. Therefore, MIOX

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may be responsible for the MI depletion found in diabetic complications.

MIOX is predominantly expressed in the kidney, nerves and liver [9–12]. The promoter region of both the human and murine MIOX genes contain osmotic response element(s) (OREs) and carbohydrate response element(s) (ChREs). The binding activities of the nuclear factor of activated T cells 5 (NFAT5) and carbohydrate response element binding protein (ChREBP) to OREs and ChREs, respectively, significantly increase under high glucose conditions [8,13]. High glucose and osmolytes significantly increase the transcriptional activity of MIOX by in vitro luciferase assays. Furthermore, increased MIOX expression has been shown in the kidneys of diabetic mice [8]. In preliminary experiments, we have shown that human peripheral blood mononuclear cells (PBMCs) express the MIOX gene by a direct sequencing of reverse transcriptase polymerase chain reaction (RT-PCR) products and Western blotting. In related studies, we have shown that NFAT5 is involved in the regulation of AKR1B1 under high glucose conditions [14] and in the presence of an aldose reductase inhibitor (ARI), the binding activities of nuclear factor kappa B (NFκB) [15] and NFAT5 (unpublished data) to OREs in the promoter of the AKR1B1 gene were suppressed. Therefore, our hypotheses are that high glucose increases MIOX protein levels and activity which might be regulated through increased binding activities of NFAT5 to OREs and ChREBP to ChREs in the promoter region of the MIOX gene. These binding activities may be different in the PBMCs from patients with nephropathy, compared to those without complications. Inhibition of AKR1B1 may suppress MIOX protein levels and activity through reducing the production of sorbitol through the polyol pathway.

To the best of our knowledge, there has been no study that has investigated the factors involved in the regulation of the MIOX gene and enzyme activity in patients with type 1 diabetes mellitus (T1D) and nephropathy. Therefore, the aims of this study were to investigate the regulation of MIOX expression under high glucose conditions in the PBMCs from patients with T1D, with or without nephropathy.

2. Materials and methods

2.1. Subjects

The following Caucasoid subjects were included in this study: 34 patients with T1D and 9 ethnically matched healthy controls. All patients with T1D as defined by The Expert Committee On The Diagnosis And Classification Of Diabetes Mellitus [16] had attended the Diabetes Clinic, Derriford Hospital, Plymouth. The study was approved by the Local Research Ethical Committee, and informed consent was obtained from all subjects. The criteria for diabetic microvascular complications have been published previously [17].

2.1.1. Uncomplicated

Patients ($n = 14$) have been diagnosed with T1D for at least 20 years but remain free of retinopathy (fewer than five dots or blots per fundus), proteinuria (urine Albustix negative on at least three consecutive occasions over 12 months) and neuropathy (overt neuropathy was defined if there was any clinical evidence of peripheral or autonomic neuropathy).

2.1.2. Diabetic nephropaths

Patients ($n = 20$) have had T1D for at least 8 years with persistent proteinuria (urine Albustix positive on at least three consecutive occasions over 12 months or three consecutive total urinary protein excretion rates >0.5 g/24 h) in the absence of hematuria or infection on midstream urine samples. Diabetic nephropathy was always associated with retinopathy. Retinopathy was defined as more than five dots or blots per eye; hard or soft exudates, new vessels, or fluorescein angiographic evidence of maculopathy or previous laser treatment for pre-proliferative or proliferative retinopathy; and maculopathy or vitreous haemorrhage. Fundoscope was performed by both a diabetologist and an ophthalmologist.

2.2. Cell isolation and cultures

Peripheral venous blood samples (20 ml) were collected into 5% EDTA Vacutainers (Becton Dickinson, UK). The peripheral blood mononuclear cells (PBMCs) were separated by using Histopaque (Sigma, Dorset, UK) and grown in RPMI 1640 supplemented with D-glucose at a concentration of 5.5 mmol/l, 10% calf serum and 2 mmol/l L-glutamine, 100 units/ml penicillin G sodium and 100 mg/ml streptomycin sulfate with PHA-P at a concentration of 5 μg/ml in a 37 °C incubator with a controlled, humidified atmosphere of 95% air/5% CO_2. The cells were divided into three groups in 200 ml-flasks. Group 1 (normal conditions-NG): cells were cultured in the above medium (D-glucose at a final concentration of 5.5 mmol/l). Group 2 (high glucose conditions-HG): 19.5 mmol/l extra D-glucose was added into above-mentioned media (D-glucose at a final concentration of 25 mmol/l). Group 3 (aldose reductase inhibitor conditions (ARI) with HG): sorbinil (at a final concentration of 10 μmol/l), was added 3 h later after 19.5 mmol/l extra D-glucose was added to the media. All cells were incubated for 5 days. At the end of the incubation time, cells were harvested and nuclear and cytoplasmic proteins were extracted as below.

2.3. Extraction of nuclear protein and cytoplasmic proteins

Cells were collected and re-suspended in 100 μl of buffer A (10 μmol/l HEPES, pH 7.9, 1.5 mmol/l $MgCl_2$, 0.5 mmol/l dithiothreitol (DTT), 0.2% NP-40, 100 mmol/l 4-(2-aminoethyl)-bezenesulfonyl fluoride (AEBSF), 18.4 mg/ml sodium orthovanadate, 42 mg/ml sodium fluoride and 2.2 mg/ml aprotonin) and held on ice for 15 min. The resulting cell lysate was then centrifuged at 13,000 rpm for 10 min. The supernatant containing cytoplasmic proteins was transferred into a fresh tube and stored at −80 °C for Western blotting and MIOX activity assays. The nuclear pellets were re-suspended in 50 μl of buffer C (20 mmol/l HEPES pH 7.9, 25% glycerol, 0.42 mol/l NaCl, 1.5 mmol/l $MgCl_2$, 0.5 mmol/l DTT, 0.2 mmol/l EDTA, 100 mmol/l AEBSF, 18.4 mg/ml sodium orthovanadate, 42 mg/ml sodium fluoride and 2.2 mg/ml aprotonin), and incubated on ice for 10 min. After centrifugation at 13,000 rpm for 10 min the supernatant containing the nuclear protein was transferred into a fresh tube and stored at −80 °C until use in the electrophoretic mobility shift assay (EMSA). The concentrations of both nuclear and cytoplasmic proteins were determined using a Coomassie® Plus Protein Assay kit (Peribo Science Ltd., Chest, UK).

2.4. Electrophoretic mobility shift assay

NFAT5 and ChREBP probes with consensus sequence to the OER and ChRE motifs CCTCCTCCAGGAAAGCCTTTACCCTCC and GAGCACGTGACCTACCCGTGTTG GGACACGTGAGG [8,13] of the MIOX gene were labeled with [α-^{32}P] deoxy-ATP by T4 polynucleotide kinase (Amersham Pharmacia Biotech, Buckinghamshire, UK). The labeled probes along with the gel-binding buffer were incubated with 25 μg of nuclear proteins at room temperature for 20 min. The binding mixtures were resolved by electrophoresis on a 4% non-denaturing polyacrylamide gel at 100 V for 3–4 h. The gel was exposed to X-Omax photographic paper.